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Supplemental information

Newt regeneration genes regulate Wingless signaling to restore patterning in *Drosophila* eye

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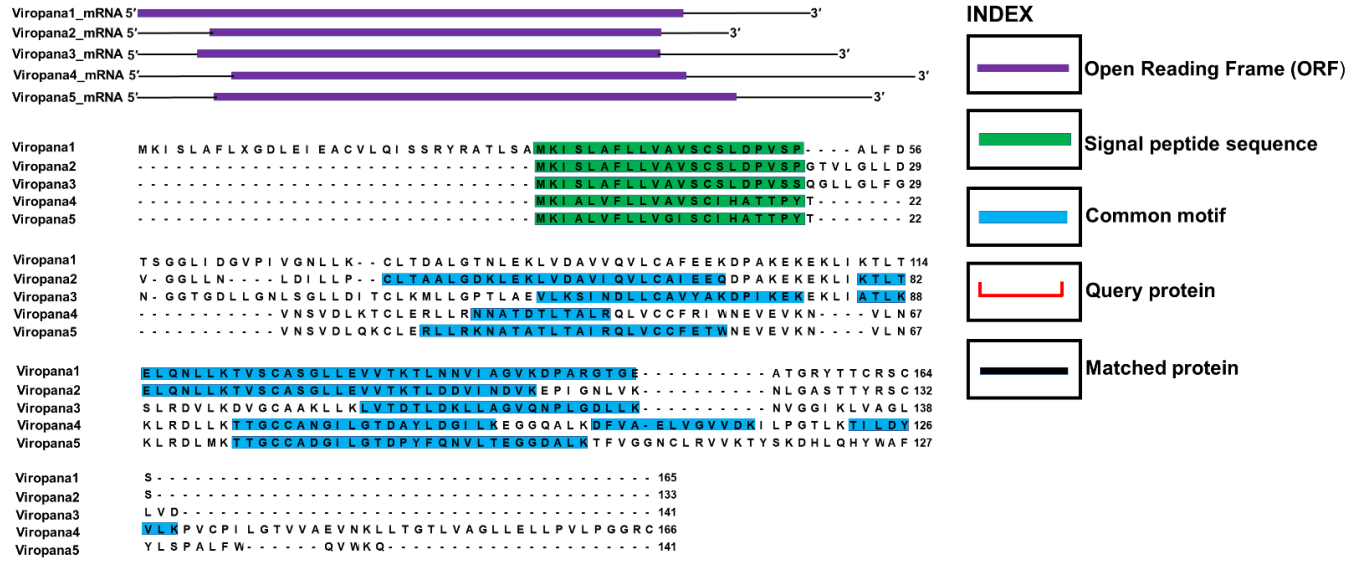
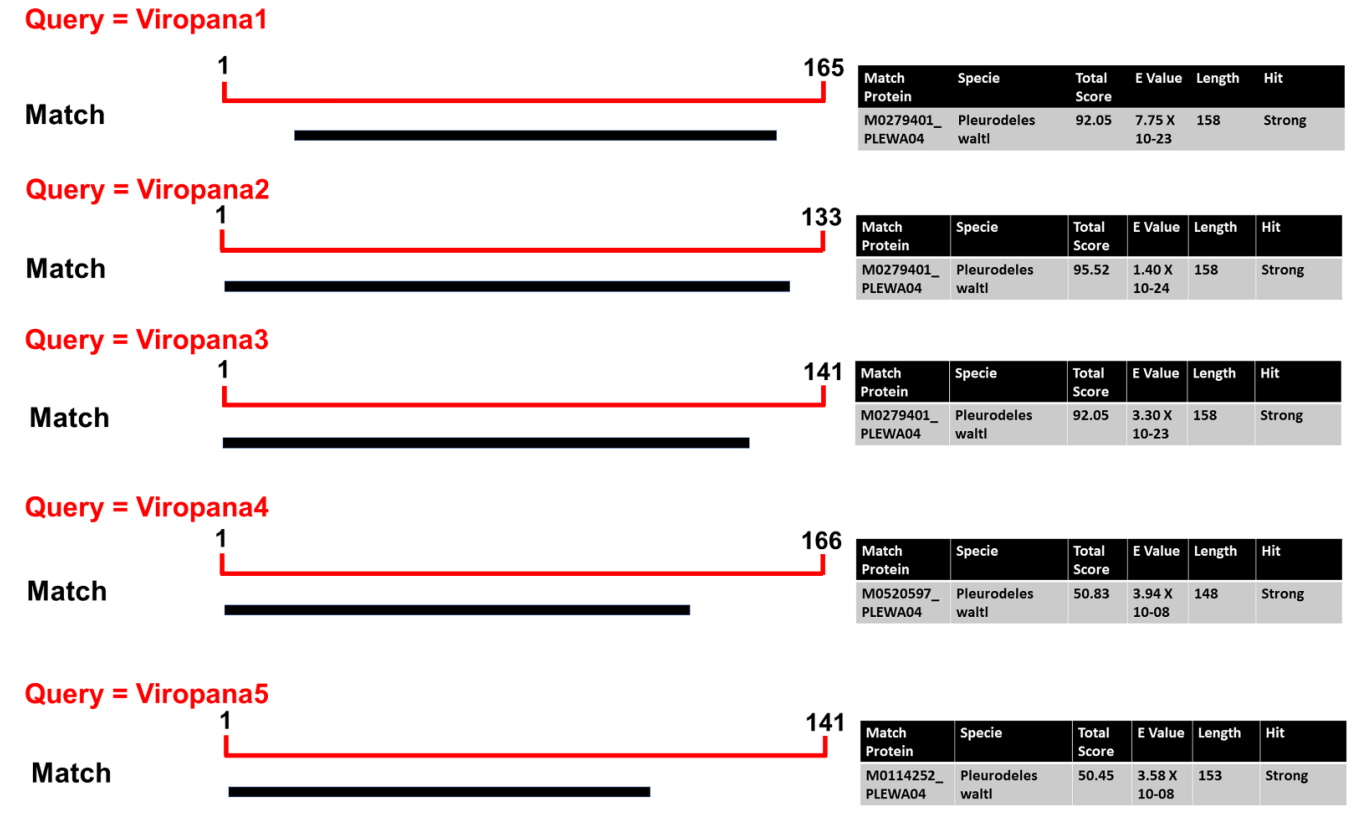
Supplemental Information

Mehta *et al.*, 2021, Figure S1



A



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M0114252_PLEWA04	Pleurodeles waltl	50.45	3.58 X 10-08	153	Strong

B

Figure S1. Identification of novel newt (*Notophthalmus viridescens*) protein family, related to Figure 1.Schematic transcript sequence that (A) includes open reading frame (ORF) in purple boxes and flanking untranslated regions (UTRs). Amino acids with significant signal peptides are marked in green, and sequences with shared motifs among the 5 genes is highlighted in blue. These proteins have missing conserved domains information. (B) These newt *"Notophthalmus viridescens"* proteins show strong match with proteins from another newt specie, *"pleurodeles waltl".*

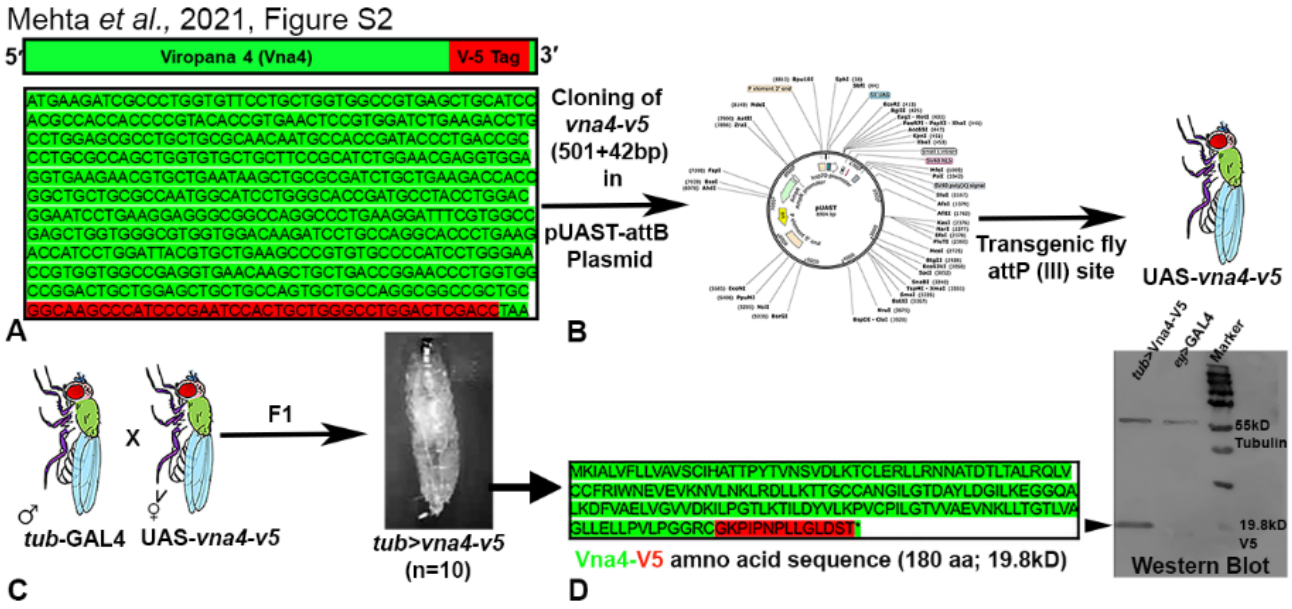

Figure S2. Validation of newt genes misexpression in the *Drosophila*, related to Figure 1. To validate the misexpression of newt gene in *Drosophila* we tagged viropana4 with (A) V5 epitope (B) Generated transgenic fly containing *vna4-V5* fused gene (C) misexpressed in the F1 progeny and (D) performed western blotting against V5 epitope. (A) The 42 bp long V5 tag is fused towards the 3' end of the 501 bp long open reading frame (ORF) of newt gene (*vna4*). (B) The ORF comprising both *vna4* and *V5* fused together as 501+42= 543 bp long ('*vna4*-V5') is cloned into the pUAST-attB plasmid. The plasmid is microinjected at a specific site (attP) on the III chromosome in the *Drosophila* to generate a transgenic fly strain: UAS–*vna4-V5*. (C) *Drosophila* strain trapped with *tub*-GAL4 enhancer is mated with transgenic *Drosophila* (*UAS–vna4-V5*) to drive *vna4-V5* misexpression ubiquitously in the (F1) progeny (*tub>vna4-V5*). The protein is extracted from the third instar larvae of F1 progeny (*tub>vna4-V5*), n=10. (D) Vna4-V5 fused protein is 166 aa (Vna4) + 14 aa (V5)= 180 aa long, which is equivalent to molecular weight of 19.8 kilodalton (kD). The Blot shows the expression of fused newt gene protein (Vna4-V5) in *Drosophila melanogaster.* The samples were loaded in the following sequence: Lane 1- *tub*>Vna4-V5, Lane 2- *ey*>GAL4 (Control), Lane 3- Molecular weight marker. Alpha tubulin is used as a loading control. Molecular weight of alpha tubulin is 55 kD. The samples were treated with anti-V5 tag antibody, and anti-α tubulin antibody. The presence of V5 band (of molecular weight 19.8 kD) in *tub>vna4-V5* lane suggests that *vna4* (newt gene) is expressed in *Drosophila melanogaster* using targeted misexpression approach.

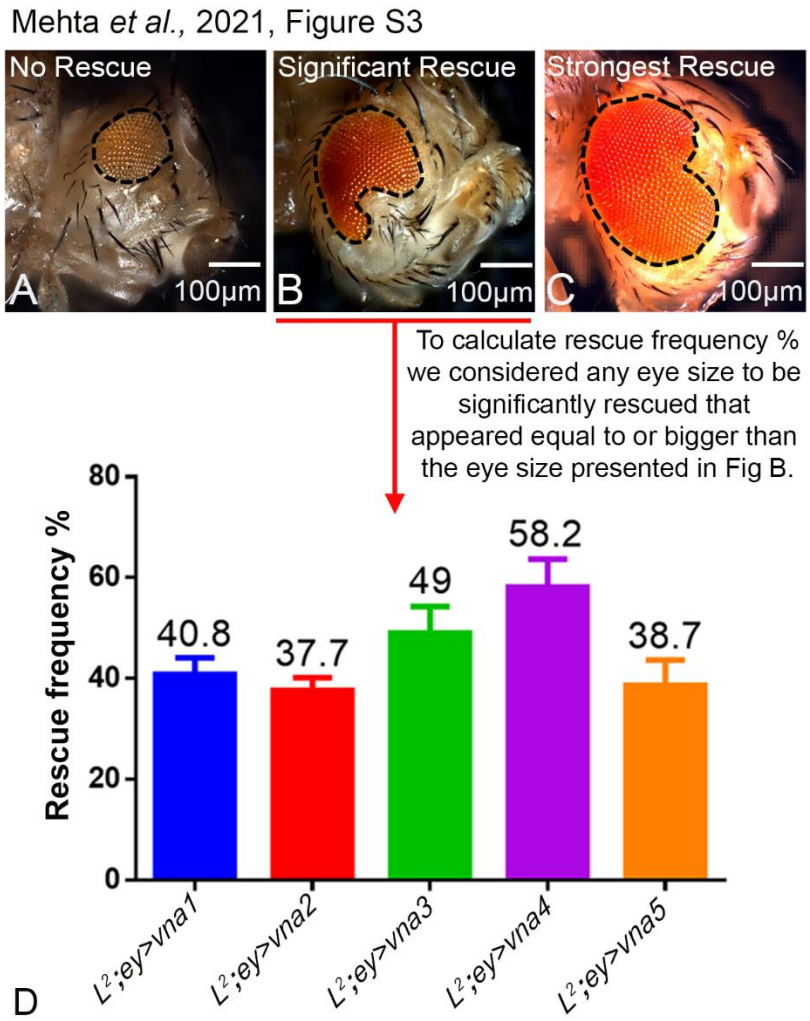

Figure S3. Rescue frequency of loss-of-ventral eye phenotype upon misexpression of respective *newt genes* in *L²mutant* background, related to Figure 1. Different *Drosophila* adult eye images as a representative example of our criteria to consider the eye as (A) No rescue (B) Significant Rescue (C) Strongest Rescue. All the Drosophila eyes that visible appeared to be similar or larger than the eye in figure B were a significant rescue, based on which Rescue frequency % was calculated (D). Rescue frequency % obtained was about 40.83% in *vna1*, 37.7% in *vna2*, 49% in *vna3*, 58.2% in *vna4*, and 38.7% in *vna5* respectively. All bar graphs show rescue frequency as average between 3 repetitions. Error bars show standard deviation (mean ± SD), and numbers above the error bar show rescue frequency in percentage. Two hundred flies were counted per repetition (200 X 3= 600) for calculating the frequency for each sample. *L²*-mutant shows 100% penetrance.

Mehta *et al.*, 2021, Figure S4

Figure S4. Unique newt candidate genes can also promote rescue in later stages of eye development, related to Figure 1. (A-C) Bright field adult eye pictures (A) Wild-type adult eye as positive control (*ey*-GAL4) (B) GMR-*hid*,GMR-GAL4 (GMR>*hid*) adult eye as negative control. Misexpression of *hid* results in a "No-eye" or highly reduced eye phenotype. (C) Misexpression of newt gene (*vna4*) in GMR-*hid*, GMR-GAL4 (GMR>*hid; vna4)* background result in a significant rescue of the "No-eye" phenotype. Thus, confirming that newt genes can also promote rescue in later stages of eye development. All the images are displayed in same polarity as dorsal domain-towards top, and ventral domain-towards bottom. Scale bar= 100 µm.

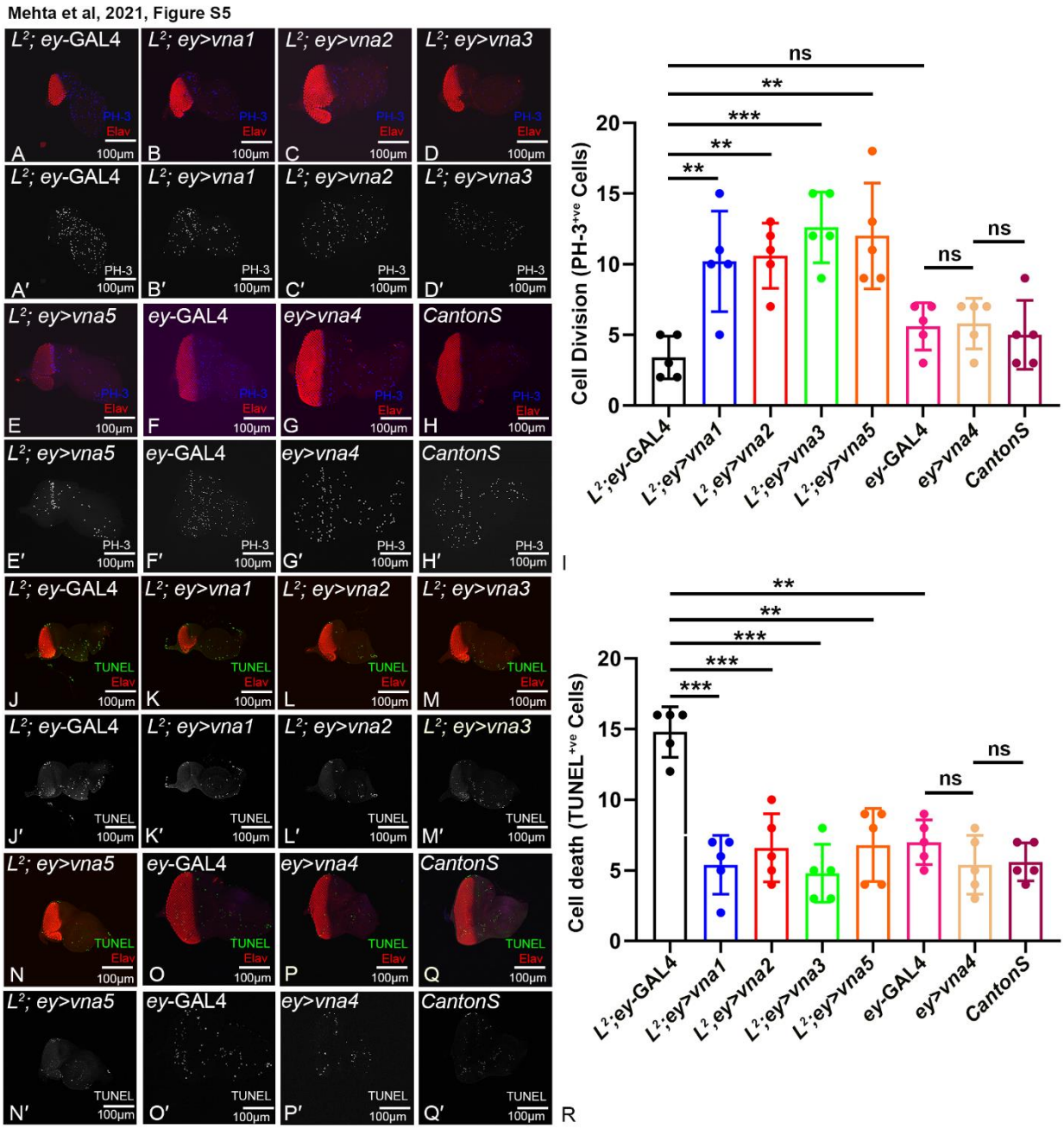

Figure S5. All the members of the unique newt gene family induce cell proliferation, and block cell death to promote rescue of *L²* mutant loss-of-ventral eye phenotype, related to Figure 3. Testing other members of the unique newt gene family we found same trend in upregulation of (A-I) cell proliferation, and (J-R) downregulation in cell death. (A-H & A′-H′) eye discs showing PH-3 as a marker to calculate rate of cell proliferation (A) *L²* mutant eye disc (B-E) *L²* mutant eye disc under the background of which newt genes (B) *vna1,* (C) *vna2*, (D) *vna3*, and (E) *vna5* are misexpressed respectively (F) Wild-type (*ey*-GAL4) eye disc with a trapped GAL4. (G) The wild type eye disc under background of which newt gene is misexpressed (*ey>vna4*) disc (H) Wild-type (*CantonS*) eye disc (I) bar graph showing increase in the cell proliferation when newt genes are misexpressed in *L²* mutant background compared to *L²* mutant fly. These results show that all the *newt genes* behave similarly and induce cell proliferation to restore the missing

cells. And thereby significantly rescuing the L^2 mutant's loss-of-ventral eye phenotype. (J-Q & J′-Q′) Eye discs showing TUNEL staining as a marker for cell death assay in (J) L^2 mutant eye disc (K-N) L^2 mutant eye disc where newt genes (K) *vna1*, (L) *vna2*, (M) *vna3,* and (N) *vna5* are misexpressed respectively (O) Wild-type (*ey*-GAL4) eye disc with a trapped GAL4. (P) The wild type eye disc under background of which newt gene is misexpressed (*ey>vna4*) disc (Q) Wild-type (*CantonS*) eye disc (R) Bar graph showing decrease in the dying cells when newt genes are misexpressed in the L^2-mutant background compared to L^2 mutant fly. These results together show that the *newt genes* apart from upregulating cell division also downregulate number of dying cells in order to restore, and rescue L^2 mutant loss-of-ventral eye phenotype. Statistical analysis was performed using student's t-test for independent samples. Sample size used for the calculation was five in number (n=5). Statistical significance was determined with 95% confidence ($p<0.05$). All bar graphs show relative fold change in cell proliferation and cell death as average between 5 samples. Error bars show standard deviation (mean ± SD), and symbols above the error bar ** signify p value <0.01, *** signify p value <0.001 and ns signify p value > 0.05 respectively. All the images are displayed in same polarity as dorsal domain-towards top, and ventral domain-towards bottom. Scale bar= 100 μm.

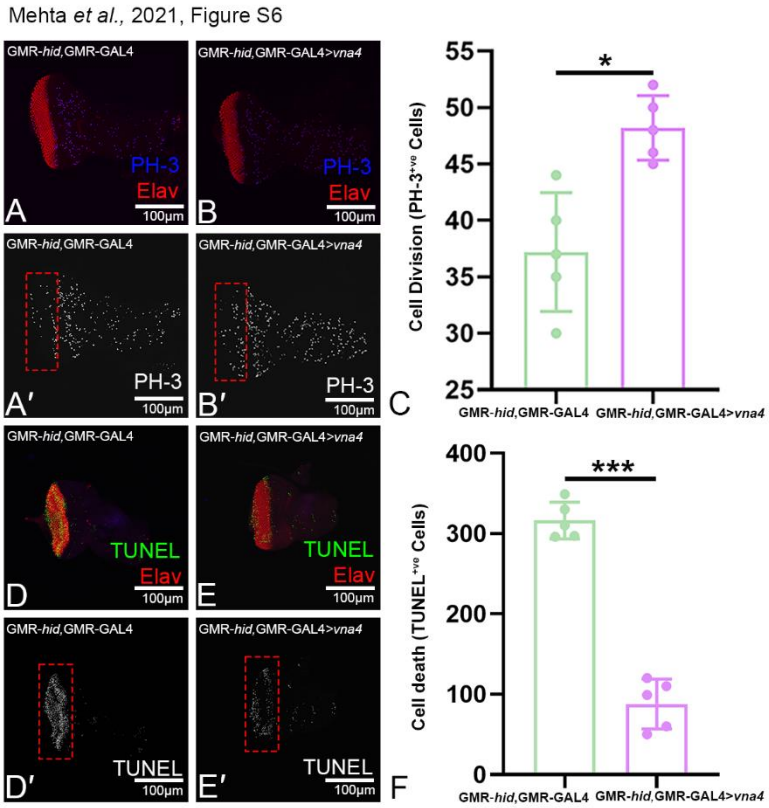

Figure S6. Newt genes induce cell proliferation and downregulate cell death to promote partial restoration of eye loss caused by overexpression of *hid* (GMR-*hid*, GMR-GAL4), related to Figure 3. Testing newt gene potential to rescue GMR>*hid* eye loss phenotype we found same trend in upregulation of (A-C) cell proliferation, and (D-F) downregulation in cell death. (A,B & A′,B′) eye discs showing PH-3 as a marker to calculate number of dividing cells in the region of interest (ROI) marked by red dotted boundary (A) GMR>*hid* eye disc (B) GMR>*hid* eye disc under the background of which newt genes *vna4 is* misexpressed (GMR-*hid*, GMR-GAL4>*vna4*) (C) bar graph showing increase in the number of proliferating cells when newt genes are misexpressed in the GMR>*hid* mutant background. These results show that *newt genes* induce cell proliferation to restore the missing cells. And thereby significantly rescuing the GMR>*hid* eye loss phenotype. (D,E & D′,E′) Eye discs showing TUNEL staining as a marker for dying cells in the region of interest (ROI) marked by red dotted boundary in (D) GMR>*hid* eye disc (E) GMR>*hid* eye disc under the background of which newt genes *vna4 is* misexpressed (GMR-*hid*, GMR-GAL4>*vna4*) (F) bar graph showing robust decrease in the number of dying cells when newt genes are misexpressed in the GMR>*hid* mutant background. These results together show that the *newt genes* apart from upregulating cell division also downregulate number of dying cells in order to restore, and rescue GMR>*hid* loss of eye phenotype. Statistical analysis was performed using student's t-test for independent samples. Sample size used for the calculation was five in number (n=5). Statistical significance was determined with 95% confidence ($p<0.05$). All bar graphs show change in rate of cell proliferation and cell death as average between 5 samples. Error bars show standard deviation (mean ± SD), and symbols above the error bar * signify p value <0.05, *** signify p value <0.001 respectively. All the images are displayed in same polarity as dorsal domain-towards top, and ventral domain-towards bottom. Scale bar= 100 µm.

Mehta et al, 2020, Figure S7

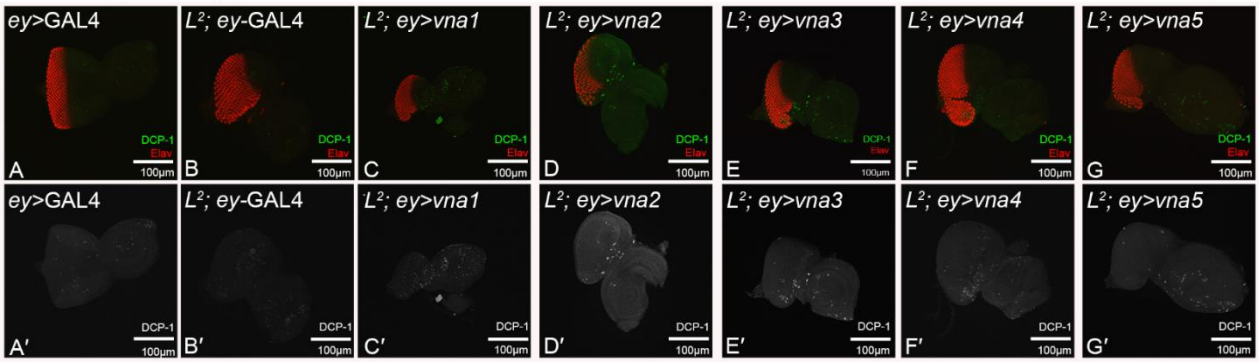

Figure S7. Downregulation in dying cells by misexpression of newt genes in *L²*-mutant background, related to Figure 3. (A-G) Eye discs marked by cleaved *Drosophila* DCP-1 antibody. *Drosophila* cell death caspase (DCP-1) is *Drosophila* homologue of human caspase 3 that marks dying cells. (A′-G′) are single channel images showing number of dying cells in respective eye discs. Results again show that misexpressing newt genes downregulate number of dying cells in *L²* mutant background. All the images are displayed in same polarity as dorsal domain-towards top, and ventral domain-towards bottom. Scale bar= 100 µm.

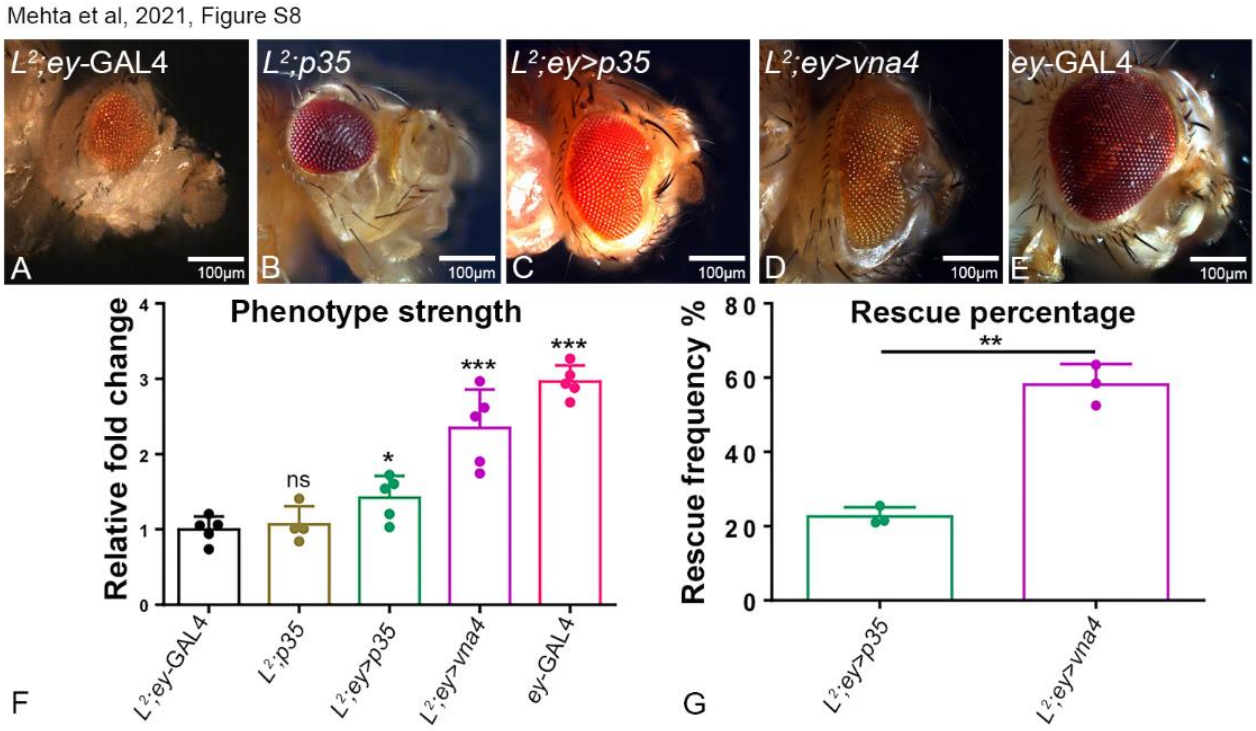

Figure S8. Comparison of rescue of *L²* mutants by baculo-virus P35 mediated downregulation of cell death with misexpression of newt gene *vna4,* related to Figure 3. (A) *L²* mutant eye (*L²; ey*-GAL4) (B) *L²* mutant adult eye that contains *p35* as a transgene (*L²/+; p35*) (C) *L²* mutant adult eye in which p35 is misexpressed using ey-GAL4 (*L²/+; ey>p35*). (D) *L²* mutant adult eye in which *vna4* newt gene is misexpressed (*L²/+; ey>vna4*). (E) Wild type adult eye (*ey*-GAL4) as positive control. (F) Phenotype strength (eye size) between the samples. (G) Comparison of loss-of-ventral eye phenotype rescue frequency induced by *p35* misexpression (*L²/+; ey>p35*) with the one induced by *vna4* (newt gene) misexpression (*L²/+; ey>vna4*). Both misexpressed in the *L²* mutant background. Note that *L²* mutants loss-of-ventral eye phenotype rescued by anti-apoptotic gene, *p35* (*L²/+; ey>p35*) are weaker in strength, and rescue frequency is significantly less when compared to the *L²* mutant fly eye in the background where newt gene is misexpressed (*L²/+; ey>vna4*). Statistical analysis was performed using student's t-test for independent samples. Statistical significance was determined with 95% confidence ($p<0.05$). In case of (F) phenotype strength all bar graphs show relative fold change in the eye size as average between 5 samples. In case of (G) rescue frequency percentage. All bar graphs show rescue frequency as average between 3 repetitions. Two hundred flies were counted per repetition for calculating the frequency for each sample. Error bars show standard deviation (mean ± SD), and symbols above the error bar * signify p value <0.05, ** signify p value <0.01, *** signify p value <0.001, and ns signify p value >0.05 respectively. *L²*-mutant shows 100% penetrance. All the images are displayed in same polarity as dorsal domain-towards top, and ventral domain-towards bottom. Scale bar= 100 µm.

Mehta et al, 2021, Figure S9

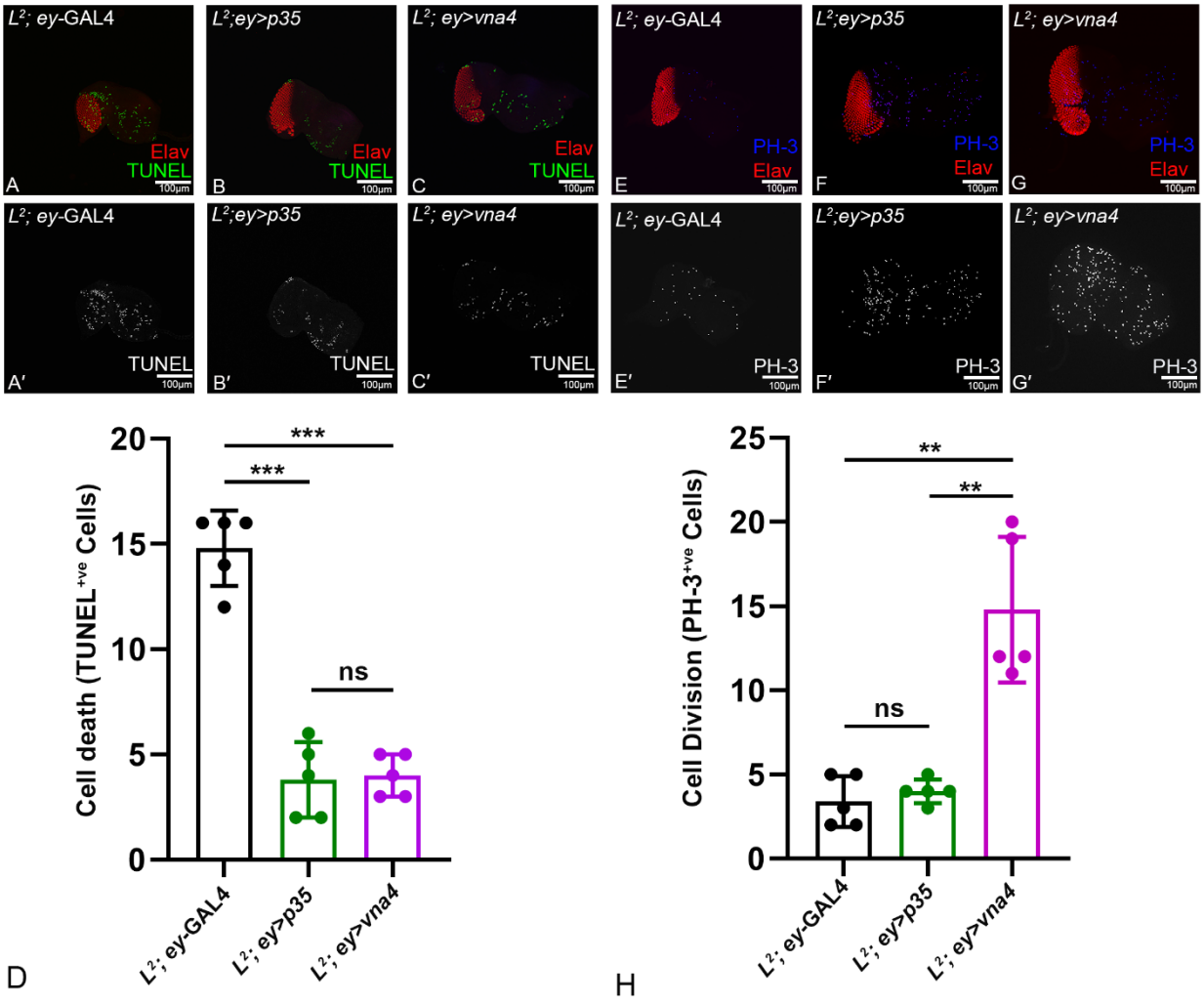

Figure S9. Comparison of cell death and cell proliferation rate among *L²* mutant (*L²*; *ey*-GAL4) vs *L²/+; ey>p35*, and *L²/+; ey>p35 vs L²*; *ey>vna4*, related to Figure 3. Testing if newt gene favor cell proliferation more than cell death in order to restore the missing structures we compared rate of cell death between (A) *L²*; *ey*-GAL4, vs (B) *L²/+; ey>p35*, and (B) *L²/+; ey>p35* vs (C) *L²*; *ey>vna4* and similarly cell proliferation between (E) *L²*; *ey*-GAL4, vs (F) *L²/+; ey>p35*, and (F) *L²/+; ey>p35* vs (G) *L²*; *ey>vna4* (C) Bar graph show comparative change in the number of dying cells among samples. (H) Bar graph show comparative change in the cell division among samples. Statistical analysis was performed using student's t-test for independent samples. Sample size used for the calculation was five in number (n=5). Statistical significance was determined with 95% confidence ($p<0.05$). All bar graphs show change in rate of cell proliferation and cell death as average between 5 samples. Error bars show standard deviation (mean ± SD), and symbols above the error bar ns signify p value >0.05, *** signify p value <0.001 respectively. All the images are displayed in same polarity as dorsal domain-towards top, and ventral domain-towards bottom. Scale bar= 100 µm.

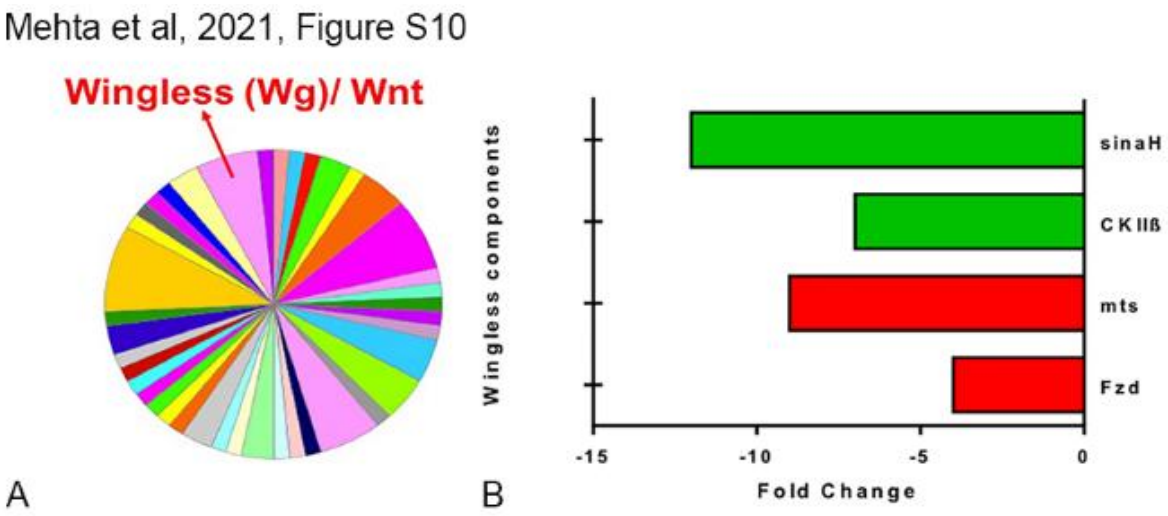

Figure S10. Misexpression of newt genes affect evolutionarily conserved Wg pathway components, related to Figure 4. Next generation RNA sequencing for wild-type *Drosophila* samples collected at third-instar larval stage under the background of which novel *newt genes* were misexpressed revealed that **(A)** Wingless/ Wnt was one of the important evolutionary conserved pathway that was reported to be differentially regulated by misexpressing newt genes ubiquitously in the developing *Drosophila*. **(B)** The components of the Wingless/ Wnt pathway that are significantly affected include (1) *frizzled* (*fz)* (principal receptors for the Wnt family of ligands) showed 4-fold downregulation, (2) *microtubule star* (*mts*) (encodes the catalytic subunit of protein phosphatase 2A) showed 9-fold downregulation, (3) *casein kinase II beta* (*CKIIβ*) (component of β-catenin degradation complex) showed 7 fold downregulation, and (4) *sina homologue* (*sinaH*) (codes for E3 ubiquitin-protein ligase) showed 12 fold downregulation. The red bars represent promoters of Wg pathway, and green bar represent inhibitors of Wg pathway.

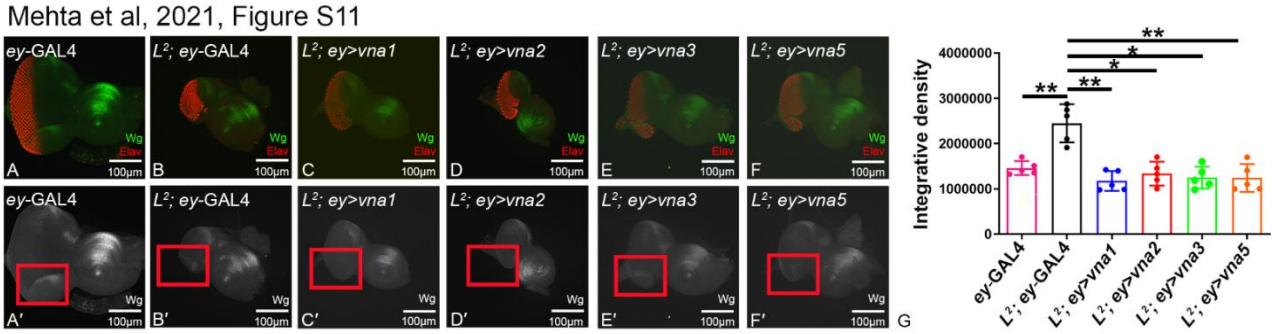

Figure S11. All the newt gene family members' downregulate Wg to promote rescue of *L²*-mutant loss-of-ventral eye phenotype, related to Figure 4. (A-F) eye disc showing wg staining in green, and Elav in red (A′-F′) are single filter confocal images showing Wg staining as grey image. The red window is the area of interest that is utilized to calculate integrative density (intensity plot). The bar graph represent the Wg intensity at the ventral margin (within the red box) in (G) Bar graphs displaying intensity plots for Wg expression of representative samples. Results clearly show the same trend of significant downregulation in Wg expression when newt genes (C, C′) *vna1*, (D, D′) *vna2*, (E, E′) *vna3*, (F, F′) *vna5* are misexpressed under the *L²* mutant background compared to the (B, B′) *L²* mutant fly. (A , A′) Is a wild-type eye disc showing normal level of Wg staining. Statistical analysis was performed using student's t-test for independent samples. Statistical significance was determined with 95% confidence ($p<0.05$). All bar graphs show integrative density as a scale to measure Wg intensity for each sample represented as the average between 5. Error bars show standard deviation (mean ± SD), and symbols above the error bar * signify p value <0.05, and ** signify p value <0.01 respectively. All the images are displayed in same polarity as dorsal domain-towards top, and ventral domain-towards bottom. Scale bar= 100 µm.

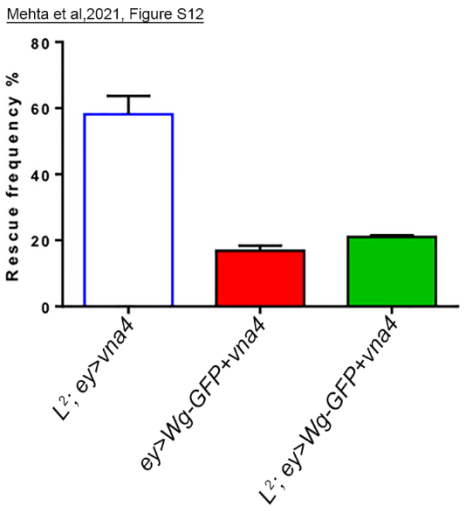

Figure S12. Percentage rescue frequency of eye-loss phenotype, related to Figure 5. Rescue frequency was about (avg) 58.2% when *vna4* alone is misexpressed in the L^2 mutant background. Rescue frequency generated by *vna4* is low when *wg* is misexpressed both in wild-type background (+/+*; ey>wg-GFP+vna4*, rescue frequency= 16.8), and under L^2-mutant background (L^2/+*; ey>wg-GFP+vna4*, rescue frequency= 21). All bar graphs show rescue frequency as average between 3 repetitions. Error bars show standard deviation (mean ± SD). Two hundred flies were counted per repetition for calculating the frequency for each sample. L^2 mutant shows 100% penetrance.

Mehta et al, 2021, Figure S13

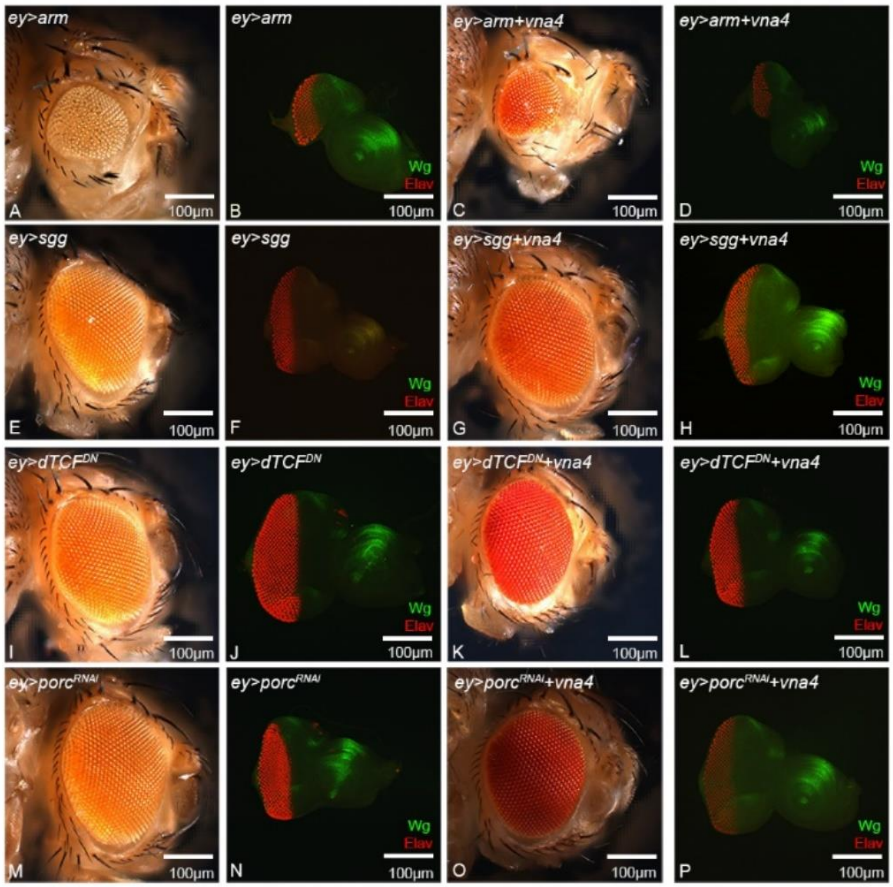

Figure S13. Modulation of positive and negative regulators of Wingless under wild type background, Related to Figure 6. Misexpression of (A, B) *arm* alone (+/+*; ey>arm*) (C, D) *arm* and *vna4* (+/+*; ey>arm+vna4*) (E,F) *sgg* (+/+*; ey>sgg*) (G, H) *sgg* and *vna4* (+/+*; ey>sgg+vna4*) (I,J) *dTCF*^{DN} (+/+*; ey> dTCF*^{DN}) (K, L) *dTCF*^{DN} and *vna4* (+/+*; ey>* dTCF^{DN}+*vna4*) (M, N) *porc*^{RNAi} (+/+*; ey> porc*^{RNAi}). (O, P) *porc*^{RNAi} and *vna4* (+/+*; ey> porc*^{RNAi}+*vna4*). Misexpression of positive regulator of Wg like *arm* cause eye-loss phenotype, and misexpressing newt genes e.g., *vna4* interact with the positive regulator of Wg pathway e.g., *arm* causing partial rescue of eye-loss phenotype in the wild type background. Misexpression of newt gene along with the negative regulators of Wg generates normal eye phenotype. Signifying that newt genes do not cause overgrowth, and they promote phenotypic transition effect only when there is the need to rescue eye-loss phenotype. Elav staining is shown in red. Wg in green. All the images are displayed in same polarity as dorsal domain-towards the top, and ventral domain-towards the bottom. Scale bar= 100 µm.

Mehta et al, 2021, Figure S14

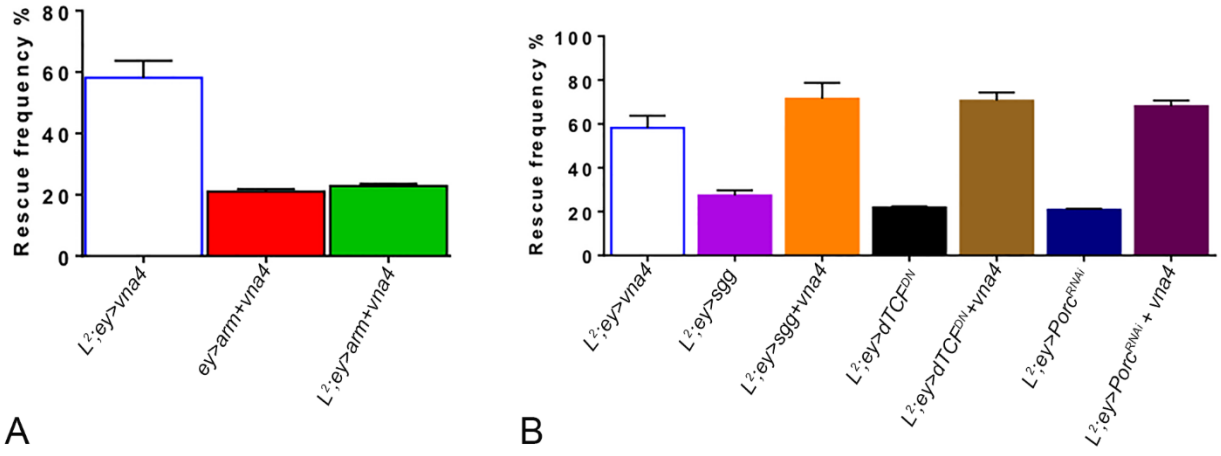

Figure S14. Percentage rescue frequency of *L²* mutant loss-of-ventral eye phenotype, related to Figure 6. (A) Rescue frequency was about 58.2% when *vna4* alone is misexpressed under the *L²*-mutant background. Rescue frequency generated by *vna4* is greater compared to when *vna4* is misexpressed along with *arm* both in the wild type background (+/+*; ey>arm+vna4,* rescue frequency= 21), and in *L²* mutant background (*L²*/+*; ey>arm+ vna4,* rescue frequency= 22.8). (B) In case of negative regulators misexpressed under the *L²*-mutant background rescue frequency is (*L²*/+*; ey> sgg,* rescue frequency=27.3), (*L²*/+*; ey> dTCF^{DN},* rescue frequency=21.8), (*L²*/+*; ey> porc*^{RNAi}, rescue frequency=20.8). All the values are below the rescue frequency obtained when *vna4* alone is misexpressed (*L²*/+*; ey> vna4*, rescue frequency=58.2). Moreover, rescue frequency dramatically increases when *vna4* is added to the negative regulators, (*L²*/+*; ey>sgg + vna4,* rescue frequency=71.5), (*L²*/+*; ey>* dTCF^{DN} + *vna4*, rescue frequency=70.6), (*L²*/+*; ey> porc*^{RNAi} + *vna4*, rescue frequency =68.1). Signifying a strong interaction between newt gene and Wg signaling pathway components. All bar graphs show rescue frequency as average between 3 repetitions. Error bars show standard deviation (mean ± SD). Two hundred flies were counted per repetition for calculating the frequency for each sample. *L²* mutant shows 100% penetrance.

Table S1. Rescue frequency calculated for respective *Drosophila* stocks, and genetic crosses involved to achieve selective genotype, Related to STAR Methods.

Genetic cross	Selected genotype	Total flies counted	Percentage frequency(In triplicate)
yw; L^2/CyO*; ey*-GAL4/*ey*-GAL4 *X yw; UAS-vna1 /S-T*	*L^2/+; ey*-GAL4/*vna1*	200*3=600	40.8
yw; L^2/CyO*; ey*-GAL4/*ey*-GAL4 *X UAS-vna2 /S-T*	*L^2/+; ey*-GAL4/*vna2*	200*3=600	37.7
yw; L^2/CyO*; ey*-GAL4/*ey*-GAL4 *X UAS-vna3 /S-T*	*L^2/+; ey*-GAL4/*vna3*	200*3=600	49
yw; L^2/CyO*; ey*-GAL4/*ey*-GAL4 *X UAS-vna4 /S-T*	*L^2/+; ey*-GAL4/*vna4*	200*3=600	58.2
yw; L^2/CyO*; ey*-GAL4/*ey*-GAL4 *X UAS-vna5 /S-T*	*L^2/+; ey*-GAL4/*vna5*	200*3=600	38.7
yw; L^2/CyO;UAS-*p35*/Tb X +/+; *ey*-GAL4/*ey*-GAL4	*L^2/+; ey*-GAL4/*UAS-p35*	200*3=600	22.7
yw; UAS-wg/CyO; UAS-vna4/UAS-vna4 X +/+; ey-GAL4/*ey*-GAL4	wg/+ *; UAS-vna4/ey*-GAL4	200*3=600	16.8
yw; UAS-wg/CyO; UAS-vna4/UAS-vna4 X L^2/CyO; ey-GAL4/*ey*-GAL4	wg/ *L^2; UAS-vna4/ey*-GAL4	200*3=600	21
yw; UAS-arm/CyO; UAS-vna4/UAS-vna4 X +/+; ey-GAL4/*ey*-GAL4	*arm/+ ; vna4/ey*-GAL4	200*3=600	21
yw; UAS-arm/CyO;UAS-vna4/UAS-vna4 X L^2/CyO;ey-GAL4/ *ey*-GAL4	*arm/ L^2; vna4/ey*-GAL4	200*3=600	22.8
yw; UAS-sgg/CyO; TM-3/Tb X L^2/CyO; ey-GAL4/*ey*-GAL4	*sgg/ L^2; TM-3/ey*-GAL4	200*3=600	27.3
yw; UAS-sgg/CyO; UAS-vna4/vna4 X L^2/CyO; ey-GAL4/*ey*-GAL4	*sgg/ L^2; vna4/ey*-GAL4	200*3=600	71.5
yw; UAS-dTCF^{DN}/CyO; TM-3/Tb X L^2/CyO; ey-GAL4/*ey*-GAL4	*dTCF^{DN}/ L^2; TM-3/ey*-GAL4	200*3=600	21.8
yw; UAS-dTCF^{DN}/CyO; UAS-vna4/UAS-vna4 X L^2/CyO; ey-GAL4/*ey*-GAL4	*dTCF^{DN}/L^2; vna4/ey*-GAL4	200*3=600	70.6
yw; UAS-porc^{RNAi}/CyO; TM-3/Tb X L^2/CyO; ey-GAL4/*ey*-GAL4	*porc^{RNAi}/ L^2; TM-3/ ey*-GAL4	200*3=600	20.8
yw; UAS-porc^{RNAi}/CyO; vna4/vna4 X L^2/CyO; ey-GAL4/*ey*-GAL4	*porc^{RNAi}/ L^2; C4/ ey*-GAL4	200*3=600	68.1

*S-T (SM6TM6b-Tb) is a linked balancer on chromosome II and Chromosome III